RESEARCH COMMUNICATION

Rationally designed selective inhibitors of trypanothione reductase

Phenothiazines and related tricyclics as lead structures

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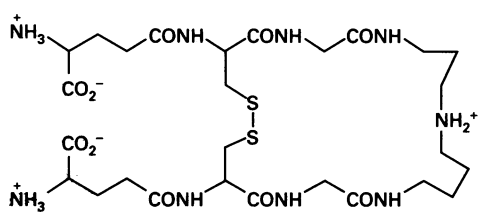
Trypanothione reductase, an essential component of the anti-oxidant defences of parasitic trypanosomes and *Leishmania*, differs markedly from the equivalent host enzyme, glutathione reductase, in the binding site for the disulphide substrate. Molecular modelling of this region suggested that certain tricyclic compounds might bind selectively to trypanothione reductase without inhibiting host glutathione reductase. This was confirmed by testing 30 phenothiazine and tricyclic antidepressants, of which clomipramine was found to be the most potent, with a K_i of 6 μM, competitive with respect to trypanothione. Many of these compounds have been noted previously to have anti-trypanosomal and anti-leishmanial activity and thus they can serve as lead structures for rational drug design.

INTRODUCTION

The mammalian redox defence system, based on GSSG and glutathione reductase (GR), is replaced in trypanosomes and *Leishmania* by an analogous, but distinct, system based on oxidized trypanothione (1) and trypanothione reductase (TR) [1]. Mammalian GR, an NADPH-dependent flavoenzyme, catalyses regeneration of GSH from GSSG (see eqn. 1); TR, the trypanosomal equivalent of GR, converts **1** (TSST) into the dithiol (or reduced) form, (HS)TT(SH) (eqn. 2):



Trypanothione (1)

$$\mathrm{GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+} \qquad (1)$$

$$\mathrm{TSST + NADPH + H^+ \xrightarrow{TR} (HS)T\text{–}T(SH) + NADP^+} \qquad (2)$$

The almost total mutual substrate exclusivity of these two enzymes is the basis [2] for rational design of selective inhibitors of TR in the presence of host GR. Powerful inhibition of TR, severely compromising the parasite's redox defences, may provide a drug lead. Selectivity of substrate binding for GR and TR has been probed by using substrate analogues [3,4], by site-directed mutagenesis [5–8] and X-ray diffraction studies [9,10].

Trypanothione (1) is an unlikely lead for drug design. Consequently, we are interested in simplifying TR recognition requirements relative to the TSST structure and in finding replacements for the highly charged residues. We have already discovered a hydrophobic site in the region of trypanothione binding and introduced [3] a cheap, convenient new assay substrate for TR based on it.

MATERIALS AND METHODS

Trypanothione reductase from *Trypanosoma cruzi* was isolated by means of overexpression. The gene coding for *T. cruzi* (Brazilian Silvio strain, clone X10/1) was amplified by the PCR from genomic DNA using primers designed for the *N*-terminal regions of the protein (A. Borges & A. H. Fairlamb, unpublished work; details available from A. B. on request; [11]). The resulting product was subcloned into the expression vector pBSTNAV [12] and expressed in *Escherichia coli* JM109 cells. The expressed enzyme (accounting for 13% of total protein), purified by a combination of affinity chromatography on 2′,5′-ADP–Sepharose and anion-exchange chromatography (A. Borges & A. H. Fairlamb, unpublished work; details available from A. B. on request) had specific activity identical with that of wild-type enzyme [3]. Enzyme activity was measured at 25 °C in 0.02 M-Hepes buffer, pH 7.25, containing 0.15 M-KCl, 1 mM-EDTA and 0.1 mM-NADPH [13] with an enzyme concentration of 0.3 μg·ml^{-1}. Human erythrocyte GR was assayed as described [14] in the presence of 1 mM-GSSG and 0.1 mM-NADPH in 0.1 M-potassium phosphate buffer (containing 0.2 M-KCl and 1 mM-EDTA), pH 7.0, at 25 °C. Inhibition type was assessed by the patterns of three classes of plot: $1/v$ against $1/[S_0]$ for various [I]; $1/v$ against [I] for various $[S_0]$; and $[S_0]/v$ against [I] at various $[S_0]$. The K_i values were estimated by weighted ($1/v_2$ for

Abbreviations used: GR, glutathione reductase (EC 1.6.4.2); TR, trypanothione reductase (EC 1.6.4.8); TSST, oxidized trypanothione.

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weighting) least-squares non-linear regression analysis of the data using the equation for linear competitive inhibition:

$$v = V_{max.}[S]/\{[S_0]+K_m(1+[I]/K_i)\}$$

Potential inhibitor design was guided by a computer-graphics model of TR, constructed by modification of the published co-ordinates of human erythrocyte GR [9] using the QUANTA software on a Silicon Graphics 240D workstation.

RESULTS AND DISCUSSION

The detailed binding of GSSG to human erythrocyte GR has been described in detail in an excellent study of substrate recognition [5,15]. Of particular interest for substrate-specificity distinction between GR and TR is the hydrophobic wall formed by Leu-17, Trp-21, Tyr-110, Met-113 and Phe-114 of TR [5], which corresponds to a polar region in GR, provided by Leu-33, Arg-37, Tyr-114, Asn-117 and Leu-118 of GR [15] and is responsible for binding the glycyl carboxylate groups of GSSG. As inhibitor target molecules were built by docking fragments into this region, it became evident from computer analysis that some known phenothiazine and tricyclic antidepressant drugs could be accommodated at this hydrophobic wall site, and comparison with the GR structure predicted them to be selective inhibitors of TR. Subsequent assay against recombinant TR confirmed this. Of 30 compounds screened, three typical neuroleptics/antidepressants [namely clomipramine (**2**), amitriptyline (**3**) and trifluoperazine (**4**); Table 1] studied in detail were found to be simple linear competitive inhibitors of TR with either trypanothione or *N*-benzyloxycarbonyl-L-cysteinylglycyl 3-dimethylaminopropylamide disulphide [3] as varying substrate (Table 1). Under comparable conditions (Table 1) these compounds did not inhibit human erythrocyte or yeast GR. Fig. 1 shows a typical example of the competitive inhibition. The simplest explanation of the results is that such drugs inhibit TR by occupying at least part of the TSST-binding region and are therefore selective, since GR does not recognize TSST. The drug mepacrine (quinacrine) has been shown to inhibit TR, but not GR, but this was discussed in terms of the resemblance of its side chain to a polyamine [16].

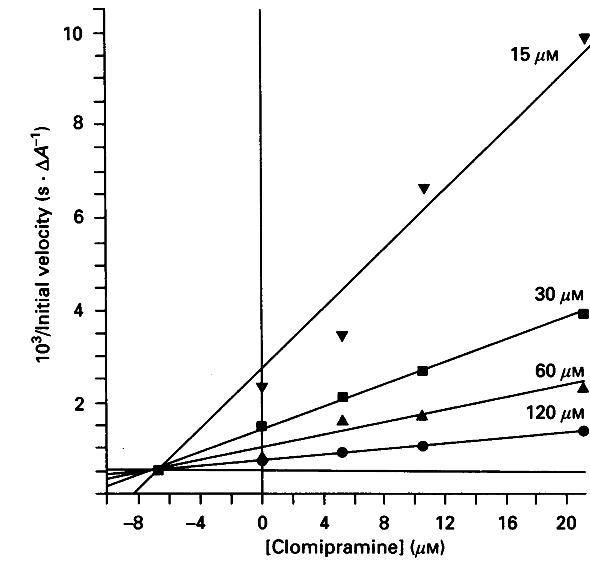
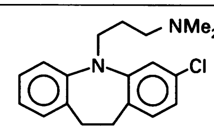
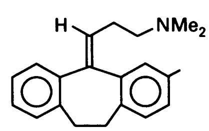
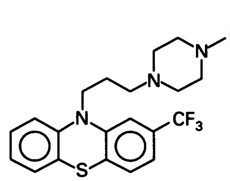


Fig 1. Dixon plot of $1/v$ against [clomipramine] for the inhibition of the TR (*T. cruzi*)-catalysed reduction of trypanothione at pH 7.25 under the conditions given in Table 1 at the trypanothione concentrations indicated against the corresponding line

Table 1. Linear competitive K_i values for the inhibition by clomipramine of recombinant TR from *T. cruzi*

Except where indicated for clomipramine, the substrate varied for all inhibitors studied was trypanothione, with the NADPH concentration being held constant at 1.0×10^{-4} M in all cases.

Compound*	Structure	K_i (μM) (mean ± S.E.M.†)
Clomipramine (**2**)		6.53 ± 0.59 6.77 ± 0.56‡
Amitriptyline (**3**)		93.6 ± 11.4
Trifluoperazine (**4**)		21.9 ± 1.7

* None of the compounds inhibited human GR when tested as described [14] at 25 °C at concentrations of 1 mM for clomipramine and amitriptyline and 0.3 mM for trifluoperazine.

† Standard error of the estimate of K_i.

‡ Using *N*-benzyloxycarbonyl-L-cysteinylglycyl 3-dimethylaminopropylamide as substrate.

Molecular modelling indicates that such molecules could bind with the tricyclic moiety lodged into the hydrophobic wall region and the aminopropyl side chain (Fig. 2*a*) extending backwards in the general direction of the active site. Binding is reasonably tight and affected by the fine structure of the central ring and/or substituents (Table 1).

Fig. 2(*b*) shows the active site from the same orientation as Fig. 2(*a*) with trypanothione docked, putting the spermidine cross-link along the hydrophobic wall. Analogues of **2**–**4**, such as chlorpromazine [17] (which also inhibits TR; results not shown), have been reported to have anti-trypanosomal activity, ascribed to interference with microtubule assembly [18], and anti-leishmanial activity, ascribed to membrane disruption [19], especially of the proton-based electrochemical gradient [20]. Other amphiphilic cationic drugs have been screened for prevention of transmission of Chagas' disease by blood transfusion [21]. Among the most potent were tricyclic antidepressants. Our findings suggest that some of the activity of the above compounds may arise from their selective effect on trypanothione metabolism, a target common to all trypanosomatids. The inhibition of TR by **2**–**4** and related structures would not have been predicted without the use of molecular graphics/modelling. The advantage of such structures as leads is that (i) many analogues of **2**–**4** have already been described, (ii) their toxicology and central-nervous-system activities are well understood and (iii) several are already

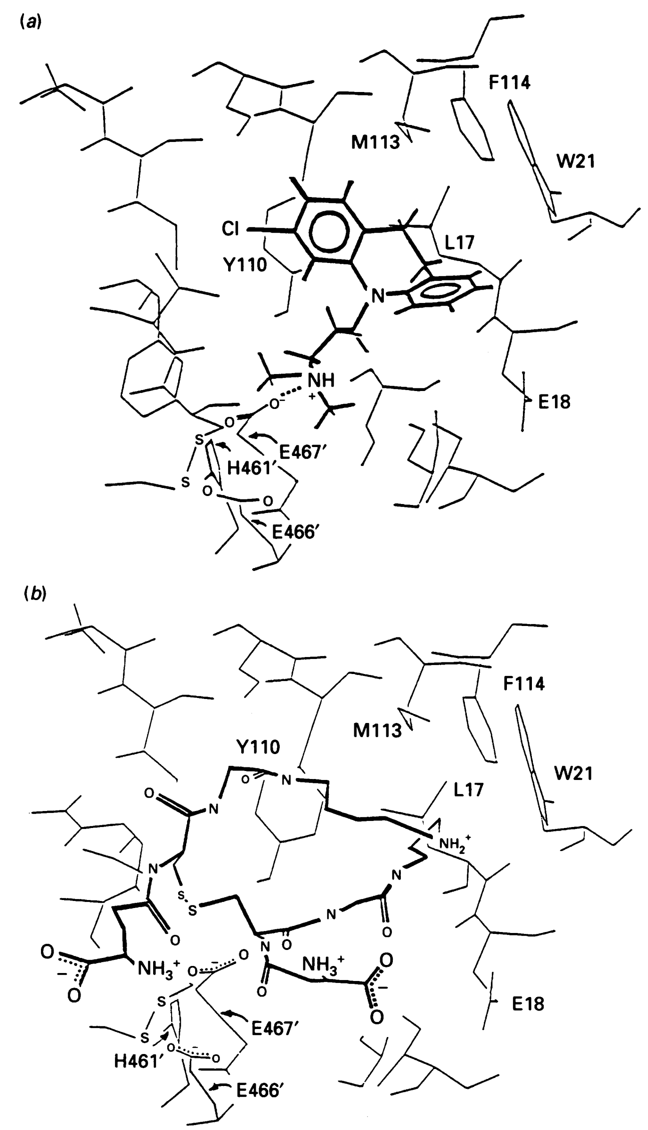


Fig 2. Docked structures of clomipramine and trypanothione in the active site of TR

(*a*) Representation of clomipramine (heavy line) docked into the active site of TR (modelled using the co-ordinates of human erythrocyte GR [9]). The hydrophobic wall formed by M113 (Met-113), F114 (Phe-114), W21 (Trp-21) and L17 (Leu-17) is closely juxtaposed to the tricyclic region of the inhibitor. The dimethylamino group of the side chain of clomipramine is shown protonated and is within salt-link distance of the carboxylate ion of the side chain of E467′ (Glu-467). The conformation of clomipramine was based on the X-ray-diffraction co-ordinates of clomipramine with suitable adjustment of the side chain [22]. Energy minimization of the enzyme–bound-ligand complex provided a structure with the tricyclic system twisted about the central ring axis through nitrogen in the opposite sense of this crystal-structure-derived conformation. In the opposite twist-sense interaction with the aromatic face of W21 looks better. (*b*) Docked representation of trypanothione bound to the active site of modelled TR from the same orientation as clomipramine shown in (*a*) with the spermidine region bound near the hydrophobic wall (M113, F114, W21 and L17). The co-ordinates of bound trypanothione were determined from those of GSSG bound to GR [9,11].

registered for use in man. Detailed structure–function studies are needed, as are detailed binding studies using X-ray diffraction and other approaches.

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